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Full Length Research Paper

Effects of additives on cordycepin production using a *Cordyceps militaris* mutant induced by ion beam irradiation

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To obtain a sustainable mutant of the medicinal mushroom *Cordyceps militaris* with a higher cordycepin production, high-energy ion beam irradiation was applied in the present study. Upon successful irradiation by a proton beam, 30 classes of 8-azaadenine and 28 classes of 8-azaaguanine resistant mutants were obtained of which 7 classes (A63-7, A63-8, A81-2, A81-6, G63-8, G81-3, G82-4) were selected as initially promising mutants using their antibacterial ability as an index of cordycepin production. Among these mutants, G81-3 had the highest cordycepin production of 6.84 g/l using optimized conditions compared to that of the control of 2.45 g/l (2.79 times higher). In addition, to explore the influences of different additives on the cordycepin production using the above mutant in a surface liquid culture, adenosine and glycine were used as additives. In the culture medium under the previously optimized conditions for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were separately added. These results revealed the highest cordycepin production of 8.57 g/l when using 6 g/l adenosine was 28.10% higher than that of the control (6.69 g/l). This is a highest report of cordycepin production until now. Similarly, the results of other concentrations also superseded the control. The time course of glucose showed that the glucose consumption for the 4 g/l adenosine was the fastest, while that of 10 g/l was the slowest with the longest culture time among all the treatments. For the same purpose, glycine was used with yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of glycine and yeast extract were fixed. Also, the glycine was separately added as 10, 20 and 30 weight percent of yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration. These results showed that the 90/10 ratio had the best cordycepin production of 6.80 g/l that was 12.40% higher versus the control (6.05 g/l). The cordycepin production of the 70/30 and 80/20 weight percent ratios were also higher than that of the control, while the others had a lower cordycepin production compared to that of the control; especially the cordycepin production with the 10, 20 and 30 weight percent yeast extracts inversely decreased in accordance with the used glycine concentration. Regarding the time course, the glucose consumption for the 40/60 weight percent ratio of yeast extract was the fastest, while that of the 30 weight percent was the slowest with the longest culture time among all the treatments. These results suggested that both the ion beam irradiation and additives had active influences on the cordycepin production and that adenosine had a much better influence than that of glycine. It was also evident that a higher concentration of both adenosine and glycine negatively affected the cordycepin production.

Key words: Ion beam, cordycepin, *Cordyceps militaris*, mutant, additives.

INTRODUCTION

It has been a difficult path to find a suitable method to increase the production of the anti-cancer agent cordycepin (3′-deoxyadenosine) from the medicinal fungus *Cordyceps militaris*. This novel bio-metabolite cordycepin has a number of valuable applications, not only against cancer, but also against some other diseases as reported by bio-

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technologists and medical researchers (Ng and Wang, 2005; Mao and Zhong, 2006). In our previous experiments, a new mutant of the *C. militaris* was obtained using ion beam irradiation technology (Das et al., 2008). In the surface culture of that mutant, it was also evident that the biosynthesis of cordycepin can be regulated by the concentrations of the components used in the culture medium, especially of the C and N sources and in addition, using some additives, as has been investigated in our previous studies (Masuda et al., 2007). The optimized medium concentrations of the C and N sources were 86.2 and 93.8 g/l, respectively, and the peak cordycepin production was 6.84 g/l. In an elaborative study to determine the strategies suitable for the extreme production of cordycepin using *C. militaris*, it was revealed that the effective additive compounds for the cordycepin production were glycine, L-aspartic acid, L-glutamine, adenine and adenosine (Masuda et al., 2007). Therefore, it was of great interest to investigate the effects of some additives like adenosine and glycine in more detail.

Adenosine or adenine is one of the end products of purine-metabolic pathway, and plays an important role as a component of DNA and RNA. In cordycepin production, the function of adenine and adenosine were the same, that is, the adenine moiety made a contribution to the cordycepin biosynthesis. Although adenine was more rapidly transported into the cells than adenosine (Masuda et al., 2007), it was insoluble in higher concentrations during medium preparation.

Similarly, glycine is an amino acid which is used in the purine-metabolic pathway as L-glutamine and L-aspartic acid. Although the cordycepin production by glycine and L-glutamine were of the same level in our previous study, it was found that the effect of glycine was able to be superimposed on that of adenine, but those of adenine and L-glutamine did not posses such a property, using a combination of two such additives (Masuda et al., 2007).

In this study, ion beam irradiation was used to generate a higher cordycepin producing *C. militaris* mutant. Furthermore, the influence of adenosine and glycine as additives was investigated in detail using a surface liquid culture with optimized medium concentrations for the higher cordycepin production.

MATERIALS AND METHODS

Fungal strain, media and stock culture

C. militaris NBRC 9787 used in the present experiments was purchased from the National Institute of Technology and Evaluation, Japan and was stored at 5℃ as the control (wild strain). It was stored on a PDA (Nissui Pharmaceutical Co., Ltd., Japan) slant at 5℃.

Mutagenesis, screening and selection of prospective mutant

The mutagenesis of the fungus *C. militaris* by the proton beam irradiation of the plate culture was carried out using the Wakasa-wan multi-purpose accelerator with a tandem injector and a synchrotron (Hitachi, Ltd., Japan) at the Wakasa-wan energy research center, Japan (Figure 1a, b) using a 200 MeV beam energy with 0.5 keV/μm LET, 250 nm penetration depth within water and 100 - 1000 Gy irradiation dose. For the growth rate measurement, a loop of mycelia from the center of the irradiated plate was inoculated on the center of a fresh 9 cm PDA plate and incubated at 25℃. The maximum and minimum diameters of the mycelial zone of each plate were measured using a vernier caliper every 3 days. The data from the 2 irradiated plates were averaged and compared to that of the control.

To obtain a higher cordycepin production, the irradiated mutants were grown in the presence of the analogue 8-azaadenine and 8-azaguanine to produce the 8-azaadenine or 8-azaguanine resistant mutants, respectively. Because, these mutants can remove the feedback inhibition cycle and thus they could increase the cordycepin production. The procedure in this experiment is as follows: A quarter of the mycelia from the irradiated and selected plate was suspended in 5 ml of physiological saline and filtered through double gauze and nylon mesh (250 μm). The filtrate was diluted to a 1/10 concentration and then spread on a Vogel's medium (using glucose instead of sucrose) agar plate supplemented with 200 mg/l of 8-azaadenine or 8-azaguanine (Vogel, 1956). Colonies that appeared on the plate after incubation at 25℃ (incubation time varied case by case) were isolated and the selected colony was inoculated on an agar slant composed of the same medium as the stock culture of the mutant that was stored as 5℃. In this way, the analogue resistant mutants were selected in order to observe the effects of the feedback inhibition cycle on the cordycepin production.

A *Bacillus subtilis* NBRC 3134 suspension with 1.5×10^5 cells/ml was then prepared for the anti-microbial test. A 0.1 ml portion of its suspension was seeded on an agar plate with Spizizen's minimum medium and 5 g/l casamino acids. Furthermore, an 8 mm disk was punched from the center of each plate on which the mutants and control strain of *C. militaris* had been cultured with the medium of the cordycepin production (Masuda et al., 2007) for 30 days at 25℃. Each disk from *C. militaris* was placed on the surface of the agar plate spread with *B. subtilis* and incubated for 24 h at 30℃. The growth-inhibiting halo toward *B. subtilis* was compared between the mutants and control. The mutants having the more distinct and larger growth-inhibiting zone than the control were utilized for the next steps (Figure 2).

Surface liquid culture using the prospective mutant

The stock culture for the control (*C. militaris*) was stored on a PDA slant at 5℃. For the 8-azaadenine or 8-azaguanine resistant mutants, a Vogel's medium agar slant supplemented with 8-azaadenine or 8-azaguanine was used for the stock culture and also stored at 5℃. The active PDA slant from the stock culture for the mutant and control was prepared by culturing for 8 days at 25℃ and then the seed culture transferred from the active slant was grown on a 9 cm PDA plate for 13 days for the control and the 8-azaadenine resistant mutant and 20 days for the 8-azaguanine resistant mutant at 25℃. The inoculum was prepared by punching out 1 cm of the PDA plate culture using a sterilized cylindrical cutter. The liquid surface culture was started by inoculating the seed disk (2 disks in one bottle) into a 500 ml culture bottle, which had a 8.5 cm diameter and 14.0 cm height with a bottleneck whose diameter and height were 4.5 and 4.0 cm, respectively. The working volume of the liquid medium was 100 ml and the bottleneck was fitted with a cotton plug during the culture. The prepared bottles were placed in an incubator maintained at 25 ± 1℃. A 1 ml portion of the medium was mainly sampled at 3 day intervals through the sampling port and filtered through a 0.45 μm membrane filter in order to remove any suspended mycelia. Just before the sampling, the

Figure 1a. Synchrotron accelerator at Wakasa-wan multi-purpose accelerator with synchroton and tandem (W-MAST).

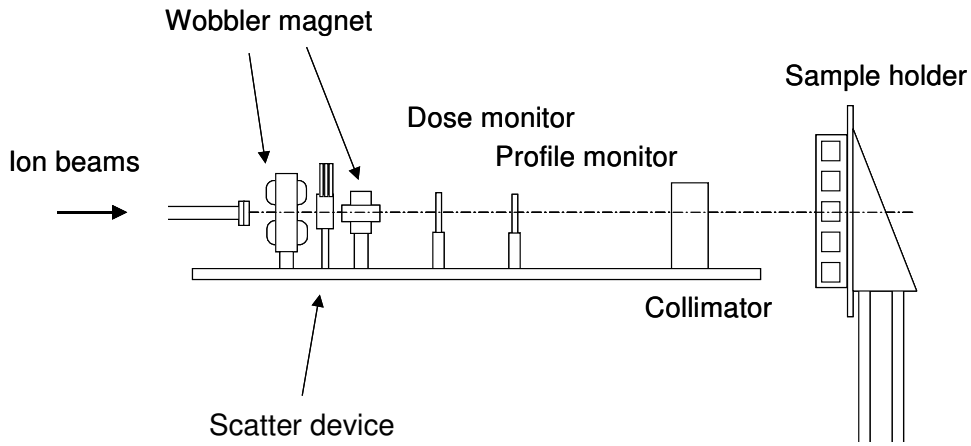

Figure 1b. Schematic diagram of ion beam irradiation apparatus for biological materials at W-MAST.

medium was gently agitated for 5 s by a magnetic stirrer. The filtrate was analyzed for cordycepin, glucose and pH. All experiments were carried out at least in duplicate and the results were then averaged. The composition of the optimized medium for the surface liquid culture used in this experiment was the same as shown in Table 1.

Experimental design for adenosine and glycine

In the culture medium using previously optimized conditions (Glucose: 86.2 and YE: 93.8 g/l) for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were separately added.

Glycine was used with the yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of glycine and yeast extract were fixed. Also, the glycine was separately added as 10, 20 and 30 weight percents of the yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration.

Analytical procedures

The cordycepin concentration was determined by an HPLC equipped with a UV detector (LC-9A system, Shimadzu Corp., Japan) and a reverse phase column (TSK-gel ODS-80Ts, Tosoh Corp., Japan). The mobile phase consisted of methanol and 0.1% phosphoric acid (2/98, v/v). The flow rate was 1.0 ml/min and the column

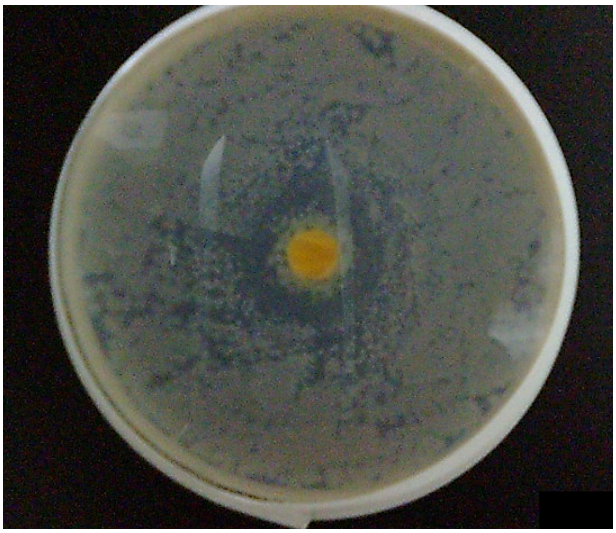

Figure 2. Photograph of growth-inhibiting zone by mutant G81-3.

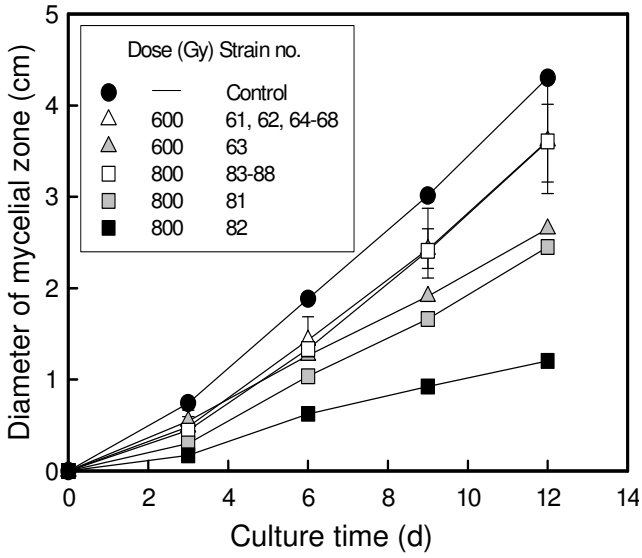

Figure 3. Time courses of the mycelial growth after 600 or 800 Gy proton beam irradiation.

Table 1. Composition of optimized medium.

Components	Concentration (g/l)
Nitrogen source	
Yeast extract	93.8
Carbon source	
Glucose	86.2
Others (diluted to 1/10 concentration of Vogel's medium)	
$NaOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$	0.28
KH_2PO_4	0.50
NH_4NO_3	0.20
$MgSO_4 \cdot 7H_2O$	0.02
$CaCl_2 \cdot 2H_2O$	0.01
Citric acid	0.46×10^{-3}
$ZnSO_4 \cdot 7H_2O$	0.50×10^{-3}
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.10×10^{-3}
$CuSO_4 \cdot 5H_2O$	0.025×10^{-3}
H_3BO_3	5.0×10^{-6}
$MnSO_4 \cdot (4–5)H_2O$	5.0×10^{-6}
$Na_2MoO_4 \cdot 2H_2O$	5.0×10^{-6}

temperature was 40°C. The chromatogram was monitored by the UV absorbance at 260 nm.

The glucose concentration was analyzed by the mutarotase-glucose oxidase (GOD) method using the glucose CII test Wako (Wako Pure Chemical Industries, Ltd., Japan). For measurement of the dry cell weight (DCW) at the end of each culture (glucose conc. < 0.008 g/l), the entire remaining content in the bottle was centrifuged at 10,000 rpm and 4°C for 20 min to separate the mycelia and then sufficiently washed with distilled water and dried at 105°C for 24 h to measure the DCW.

RESULTS

Results of ion beam irradiation

The results of the proton beam irradiation of the plate culture at doses of 200, 400, 600 and 800 Gy each showed that the growth rates of the 200 and 400 Gy doses were almost the same as that of the control (data not shown). Figure 3 shows the time courses of the mycelial growth for the irradiation doses of 600 and 800 Gy. These results indicated that some of these, which included strain nos. 63, 81 and 82, were more significantly affected than the other irradiated strains. Therefore, these strains were selected for a subsequent screening. It is noteworthy that the result of the irradiation at a dose below 200 Gy was like that of the control, while over 1000 Gy, it was unsuccessful due to lack of mycelial growth.

Upon successful irradiation by the proton beam, 30 classes of 8-azaadenine and 28 classes of 8-azaguanine resistant mutants were obtained from strain nos. 63, 81 and 82. As cordycepin inhibits the growth of *B. subtilis* (Rottman and Guarino, 1964), the *B. subtilis* growth inhibiting assays of the 58 classes of analogue resistant mutants were performed to roughly estimate the capability of the cordycepin production. Of these, 4 mutants (A63-7, A63-8, A81-2, A81-6) from the 8-azaadenine resistant mutants and 3 mutants (G63-8, G81-3, G82-4) from the 8-azaguanine resistant mutants were promising cordycepin producers. For the initial optimization, mutant no. G81-3 was selected as the best cordycepin producer among the 8-azaadenine and 8-azaguanine resistant mutants. Furthermore, the final optimized medium conditions of the C and N sources were 86.2 g/l and 93.8 g/l, respectively. The highest cordycepin production of 6.84 g/l for this mutant compared to that of the control of 2.45

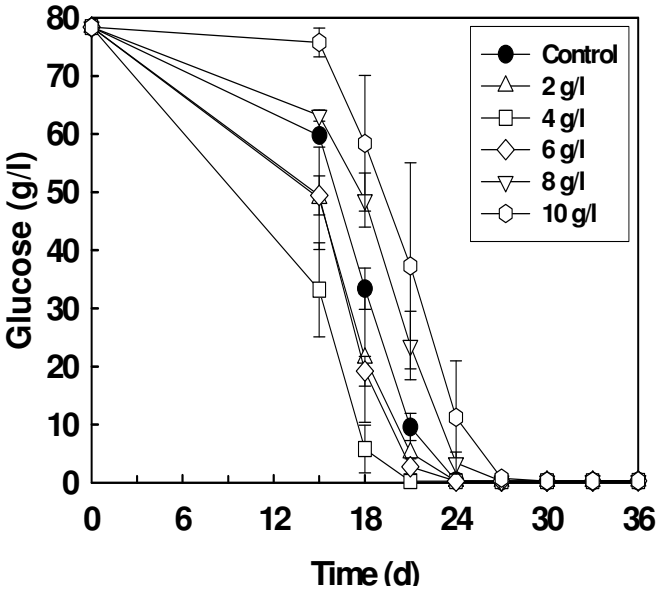

Figure 4. Time courses of glucose consumption for surface liquid culture using adenosine as an additive.

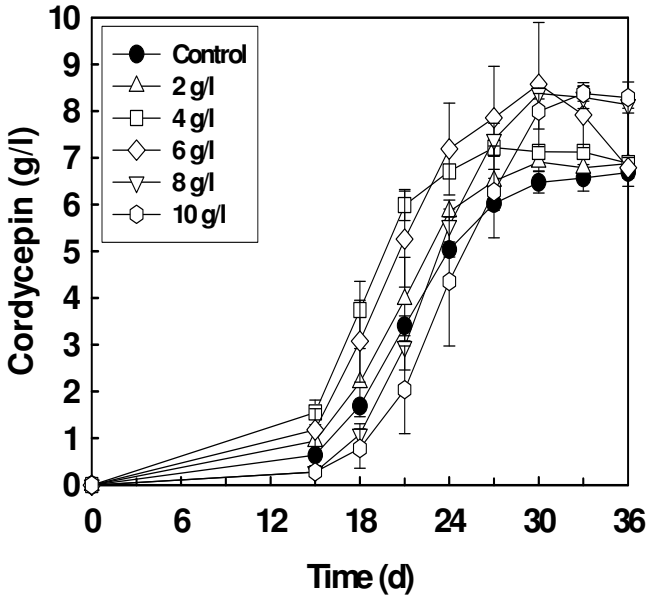

Figure 5. Time courses of cordycepin concentration for surface liquid culture using adenosine as an additive.

g/l was obtained using the central composite design (CCD) and response surface methodology (RSM) (data not shown).

Effect of adenosine on cordycepin production

In the culture medium using the previously optimized conditions for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were added separately. These results revealed that the highest cordycepin production of 8.57 g/l for the 6 g/l adenosine was 28.10% higher than that of the control (6.69 g/l) (Figure 5). This is the highest cordycepin production reported to date. Similarly, the results of the other concentrations also superseded that of the control (Figure 5). The time course of glucose showed that the glucose consumption for the 4 g/l adenosine was the fastest, while that of 10 g/l was the slowest with the longest culture time among all the treatments (Figure 4).

Effect of glycine on cordycepin production

Glycine was used with yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of the glycine and yeast extract were fixed. Also, the glycine was separately added as 10, 20 and 30 weight percent of the yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration. These results showed that the best cordycepin production of 6.80 g/l for the ratio of 90/10 was 12.40% higher than the control (6.05 g/l) (Figure 7). The cordycepin production for the ratios 70/30 and 80/20 were also higher than that of the control, while the others produced a lower cordycepin amounts compared to that of the control; especially the cordycepin production by the 10, 20 and 30 weight percents of yeast extract inversely decreased with the glycine concentration (Figure 7). Regarding the time course, the glucose consumption for the 40/60 ratio was the fastest, while for the 30 weight percent yeast extract, it was the slowest with the longest culture time among all the treatments (Figure 6).

DISCUSSION

Ion beams are expected to be widely utilized as new mutagens among the many available mutagens, because they densely deposit a high energy on a target and are locally compared to the low linear energy transfer (LET) radiations such as electrons, X-rays and γ-rays (Kraft et al., 1992) and moreover, the range of the ion beams on the target materials can be controlled (Hase et al., 1999). Thus, it is suggested that the ion beams predominately induce nuclear DNA alterations such as inversion, translocation, transversion and large deletions rather than point mutations accompanied by single- or double-strand DNA breaks with end groups and therefore, could produce various types of mutants with broad-spectrum mutations (Albert et al., 1997; Shikazono et al., 2002). There are some implications of low-energy ion implantation (~keV) (Feng et al., 2007), but the probability that the keV ion beam reaches the nuclear DNA is much lower than that of the MeV ion beam, as its penetration depth into a microorganism (~nm) is definitely much lower than

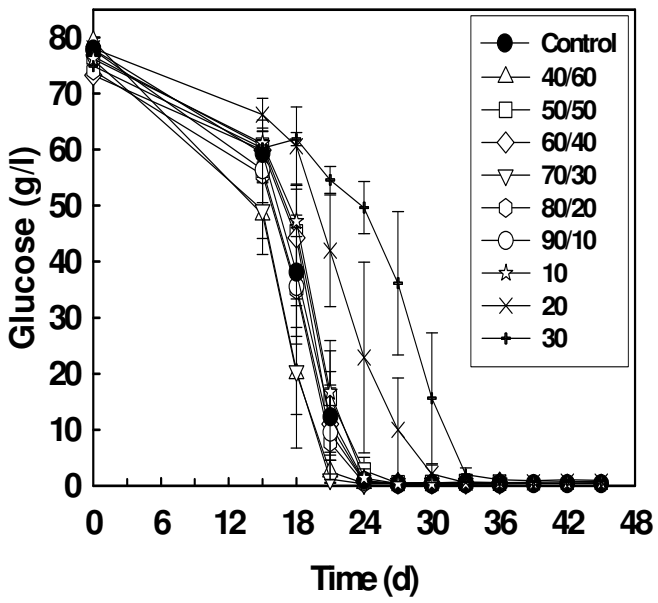

Figure 6. Time courses of glucose consumption for surface liquid culture using glycine as an additive.

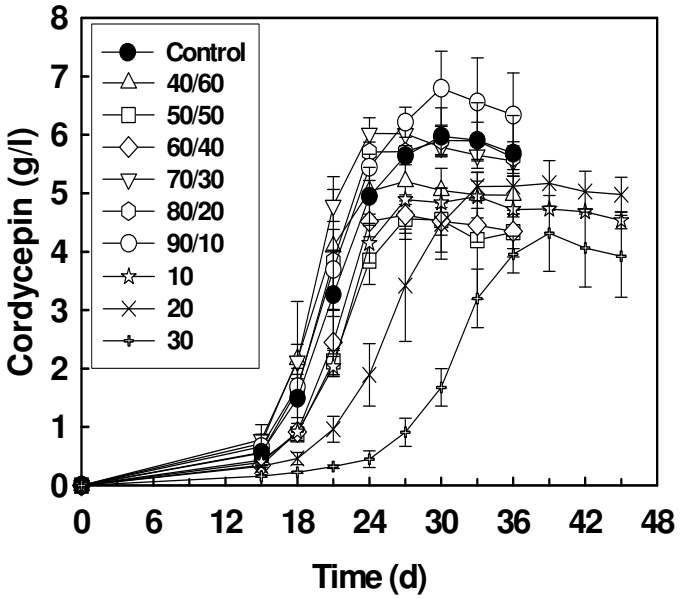

Figure 7. Time courses of cordycepin concentration for surface liquid culture using glycine as an additive.

that of the MeV ion beam.

In terms of cordycepin production by the surface liquid culture, it was found that some of the 8-azaadenine- and 8-azaguanine-resistant mutants were better producers. Generally, the levels of the end products from the biosynthesis are regulated by the feedback inhibition and thus the removal of the feedback cycle could lead to an accumulation of the end products. If a mutant can grow in the presence of 8-azaadenine, the feed-back inhibition by adenine-related compounds (adenine, adenosine, AMP) as well as 8- azaadenine is removed to some extent and thus the adenine-related compounds may be accumulated in the 8-azaadenine resistant mutant. Adenine or adenosine might be a direct precursor of cordycepin as reported in a previous study (Masuda et al., 2007) and therefore, the accumulation of adenine or adenosine could result in an increased cordycepin production. In the same manner, the guanine-related compounds (guanine, guanosine, GMP) might be accumulated in the 8-azaguanine resistant mutant that could increase the codycepin production, because the production of cordycepin and guanine might be related to each other (Masuda et al., 2007). Although the increased production of the end products is due to a variety of reasons, the feedback mechanism may be one of the major explainable causes of the increased cordycepin production in the present study.

The results of the present experiments suggested that both the ion beam irradiation and additives had active influences on the cordycepin production in which the adenosine had a much better influence than that of glycine. It is necessary to mention that the cordycepin production reported by a number of authors is much lower than that of our present research study (Table 2). As the structure of the nucleoside adenosine is very close to that of cordycepin (3′-deoxyadenosine), there might be the possibility of the metabolic conversion of adenosine to cordycepin by an unknown enzyme, which needs to be investigated. This result is consistent with the findings reported by Masuda et al. (2007) using the same species of fungus. In this experiment, it was also evident that a higher concentration of both the adenosine and glycine negatively affected the cordycepin production.

The mutant obtained by the proton beam irradiation showed a superior cordycepin production using the optimized conditions with the addition of the additives and therefore, it could be used in the realm of applied Industrial biotechnology for the large-scale production of cordycepin. The present research will also provide valuable information for mushroom researchers, cancer and radiation biologists, chemical engineers, biotechnologists, medical practitioners and personnel in the pharmaceutical industries.

ACKNOWLEDGEMENTS

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Table 2. Cordycepin production using *C. militaris* reported by several authors.

References	Methodology	Cordycepin production (g/l)	Productivity g/(l·d)
Mao and Zhong, 2004	Submerged culture	0.201	0.015
Mao et al., 2005	Submerged culture	0.345	0.019
Mao and Zhong, 2006	Submerged culture	0.421	0.025
Masuda et al., 2006	Submerged culture	0.640	0.032
Shih et al., 2007	Surface liquid culture	2.21	0.092
Masuda et al., 2007	Submerged & static culture	2.50	0.158
Results of optimization	Surface liquid culture	6.84	0.190 (mutant)
		2.45	0.102 (control)
This study	Surface liquid culture	8.57	0.286 (mutant)

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